



Research report

Microinjection of histamine into the cerebellar vermis impairs emotional memory consolidation in mice

A.C.L. Gianlorenço^a, A. Canto-de-Souza^{b,c,d}, R. Mattioli^{a,*}^a Laboratory of Neuroscience, Physiotherapy Department, Center of Biological Sciences and Health, Federal University of Sao Carlos, Rod. Washington Luis, Km 235, 13565-905, Sao Carlos, Brazil^b Psychobiology Group, Department of Psychology/CECH, Federal University of Sao Carlos, Brazil^c Interinstitutional Graduate Physiological Science, UFSCar- UNESP, Rod. Washington Luis, Km 235, 13565-905, Sao Carlos, Brazil^d Institute for Neuroscience & Behavior-IneC, USP, Ribeirão Preto, SP, 14040-901, Brazil

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ABSTRACT

The biogenic amine histamine is an important neurotransmitter in the central nervous system that has been implicated in learning and memory processes. Experimental evidence indicates that the role of the cerebellum may be more complex than the simple regulation of motor responses, and recent studies have demonstrated significant involvement of the cerebellum in emotional memory consolidation. This study investigated the effect of histamine microinjected into the cerebellar vermis on emotional memory consolidation in mice in the elevated plus-maze (EPM). The cerebellar vermis of male mice (Swiss Albino) were implanted with guide cannulae. The mice weighed between 25 and 30 g. After three days of recovery, behavioral tests in the EPM were performed on two consecutive days; the testing periods were called, Trial 1 and Trial 2. Immediately after Trial 1, the animals received microinjections of histamine in the cerebellar vermis (0.54, 1.36, 2.72, and 4.07 nmol/0.1 μ l). On both days, the test sessions were recorded to enable analysis of behavioral measures. The decrease in open arm exploration (% entries and % time spent in the open arms) in Trial 2 relative to Trial 1 was used as a measure of learning and memory. The data were analyzed using One-way Analysis of Variance (ANOVA) and Duncan's tests. The percentage of open arm entries (%OAE) and the percentage of time spent in the open arms (%OAT) were reduced in Trial 2 relative to Trial 1 for the control group; the same was true for the group that was microinjected with histamine at doses of 0.54 (%OAE and %OAT) and 1.36 nmol (%OAT). However, when the animals received histamine at doses of 2.72 and 4.07 nmol, their open arm exploration did not decrease. No significant changes were observed in the number of enclosed arm entries (EAE), an EPM index of general exploratory activity. These results suggest that there is a dose-dependent inhibitory effect of histamine microinjected into the cerebellar vermis on emotional memory consolidation.

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1. Introduction

Histamine is a biogenic amine and an important neurotransmitter–neuromodulator in the central nervous system (CNS) [13,22]. Recent evidence has clearly established that histamine and its receptors are involved in learning and memory. Numerous experiments using different learning models and

species suggest that histamine may be important for several stages of memory formation and memory retrieval during different tasks, and they emphasize the role of histamine in the physiological mechanisms of memory [1,2,23,24,39]. However, these studies have used many different behavioral tasks and produced contradictory results; for instance, both facilitatory and inhibitory effects of neuronal histamine on learning and memory have been described in animal behavior studies [1,6,15,36,46]. The mechanisms underlying these differences seem to be very complex, and the differences may be due in part to the methods used and the approaches selected in the experiments [19].

The central histaminergic nervous system originates from the tuberomammillary nucleus of the hypothalamus, and in many species, it widely innervates almost the whole brain including the cerebellum and other subcortical motor structures [38]. Previous studies have shown that the histamine-containing fibers project from the tuberomammillary nucleus to the cerebellar cortex and

Abbreviations: CNS, central nervous system; EPM, elevated plus-maze; OAE, open arm entries; OAT, open arm time; %OAE, percentage of open arm entries; %OAT, percentage of open arm time; EAE, enclosed arm entries; EAT, enclosed arm time; %EAT, percentage of enclosed arm time; CT, central area time; SAP, stretched-attend postures.

* Corresponding author at: Physiotherapy Department, Federal University of Sao Carlos, Rodovia Washington Luis, 235, Bairro Monjolinho, CEP: 13565-905, São Carlos – SP, Brazil. Tel.: +55 16 3351 8628; fax: +55 16 3361 2081.

E-mail address: mattioli@ufscar.br (R. Mattioli).

the deep cerebellar nucleus and that the highest density of histaminergic terminations is in the vermis and flocculus [21,45]. A moderately dense network of histamine fibers has been seen in the molecular and granular layers of the cerebellum in several species including humans [14]. These fibers run parallel to the Purkinje cell layer after traversing it perpendicularly. Both Purkinje cells and neurons in the nucleus interpositus have H2 receptors [37], and granule cells are excited through both H1 and H2 receptor activation [43].

The cerebellum has traditionally been considered an important subcortical motor structure, but several lines of evidence support the view that the role of the cerebellum is more complex than previously thought and includes more than just the regulation of motor responses [34]. An increasing number of studies have demonstrated its involvement in cognitive and emotional function [35]. Functional neuroimaging studies and studies of patients with cerebellar lesions have been conducted to elucidate the role of the cerebellum in the processing of emotion [35,42,44]. According to Sacchetti et al. [32], the fact that there is a functional interconnection between the cerebellar vermis and the hypothalamus, amygdala, and hippocampus suggests that the cerebellum may play a role in an integrated network regulating emotional behavior. Moreover, Ruediger et al. [29] demonstrated that fear conditioning learning is specifically correlated with the growth of feedforward inhibition connectivity in hippocampal and cerebellar circuits.

Experimental evidence indicates that the cerebellum plays a role in emotional learning. The capacity to learn and retain fear-conditioned responses was investigated in *hotfoot* mutant mice. These animals are characterized by a primary deficiency in the synapses made by the parallel fibers onto the Purkinje cells. In these mutant mice, the cerebellar dysfunction impairs learning, which suggests that these synapses are involved in fear memory consolidation [31]. Studies have related the cerebellar vermis to emotional memory consolidation. In one study, vermis inactivation caused amnesic effects after a fear conditioning task [30]. Thus, the participation of the vermis in emotional memory is independent of its role in sensory or motor processes, and the vermis may represent an interface between sensory stimuli, emotional state, and motor responses [30,34].

Studies have demonstrated the relationship between the histaminergic system and the cerebellum. Shen et al. [37] demonstrated that histamine excites the cerebellar interpositus nucleus cells via the histamine H2 receptor mechanism. They suggested that the hypothalamocerebellar histaminergic fibers may modulate neuronal activity in the cerebellum. The results of a study by Tian et al. [43], revealed that histamine excites cerebellar Purkinje cells via H2 receptors and that the histaminergic fibers may play an important role in functional aspects of the cerebellum. In spite of these investigations, there have been no reports on the cerebellar histaminergic system and learning and memory processes.

The elevated plus-maze (EPM) is an animal model test of anxiety based on rodents' natural aversion to open spaces [18]. A general aspect of EPM exploration is that animals enter and spend less time exploring the open arms [7]. The inclusion of a retest session has been made in recent years, which is consistent with the assumption that there is a learned component underlying the exploratory behavior during EPM re-exposure [8]. According to File et al. [8], after the initial exploration of the apparatus, rodents acquire, consolidate and retrieve some kind of memory related to exploration of potentially dangerous areas of the maze. Bertoglio and Carobrez [4] use Trial 1/2 protocol to show that after a single prior non-drugged experience in the maze, mice exhibit significantly reduced open arm activity in a second trial. An increase in open arm avoidance with repeated maze exposure has been observed in several studies [7,10,16] and has been used as a measure of learning and memory [12,16,36].

The present study was designed in view of these findings to investigate the action of histamine microinjected into the cerebellar vermis on emotional memory consolidation in mice using Trial 1/2 protocol in the EPM.

2. Material and methods

2.1. Animals

Male Swiss mice (Federal University of São Carlos, UFSCar, SP, Brazil) weighing 25–35 g at the beginning of the experiments were housed in polypropylene cages (31 × 20 × 13 cm) in groups of five and maintained under a 12 h light cycle (lights on at 7:00 a.m.), in a controlled environment at temperature $23 \pm 1^\circ\text{C}$ and humidity $50 \pm 5\%$. Food and drinking water were provided *ad libitum*, except during the brief test periods. All mice were experimentally naive, and the experimental sessions were conducted during the light period of the cycle (9:00–13:00 h).

2.2. Drugs

Histamine dihydrochloride (Sigma Chemical Co., USA) was prepared in a vehicle of physiological saline. Saline solution was used as an experimental control. The doses of histamine were based on previous research [28] and on pilot work in our own laboratory. The substances were coded, and the codes were unknown to the experimenter during the tests and behavioral analysis.

2.3. Surgery and microinjection

Each mouse was implanted with a single 7 mm stainless steel guide cannula (25 gauge) under ketamine chloridrate and xylazine solution anesthesia (100 mg/kg and 10 mg/kg, respectively, delivered via i.p. injection). The stereotaxic coordinates for the cerebellar vermis were 6.5 mm posterior to bregma, 0 mm lateral to the midline, and 2.0 mm ventral to skull surface [9]. The guide cannula was fixed to the skull using dental acrylic and Jeweler's screws. A dummy cannula (33 gauge stainless steel wire) was inserted into the guide cannula at the time of surgery and served to reduce the incidence of occlusion. Postoperative analgesia was provided for 3 days by adding acetaminophen (200 mg/ml) to the drinking water in a ratio of 0.2 ml acetaminophen to 250 ml water (i.e., the final concentration was 0.16 mg/ml). Saline and drug solutions were infused into the cerebellar vermis using a microinjection unit (33 gauge cannula; Cooper's Needleworks, Birmingham, UK), which extended 2.0 mm beyond the tip of the guide cannula. The microinjection unit was attached to a 5 μl Hamilton microsyringe via polyethylene tubing (PE-10), and the administration was controlled by an infusion pump (Insight Equipamentos Científicos Ltda, Brazil) programmed to deliver a volume of 0.1 μl over a period of 60 s. The microinjection procedure consisted of gently restraining the animal, inserting the injection unit, infusing the solution, and keeping the injection needle *in situ* for a further 60 s to avoid reflux. Confirmation of successful infusion was obtained by monitoring the movement of a small air bubble inside the PE-10 tubing.

2.4. Apparatus

The EPM used was similar to that originally described by Lister et al. [18]. The EPM consisted of two open arms (30 × 5 × 0.25 cm) and two enclosed arms (30 × 5 × 15 cm) connected to a common central platform (5 × 5 cm). The apparatus was made of crystal acrylic and was raised to a height of 38.5 cm above floor level. All tests were conducted under moderate illumination (77 lx) as measured on the central platform of the EPM and in an environment isolated from the rest of the room by a black protective curtain.

2.5. Experimental procedure

Three days after surgery, the animals were transported to the experimental room and left undisturbed for at least 1 h before testing to facilitate adaptation. The test was performed on two consecutive days, and the trials in the EPM were denoted: Trial 1 and Trial 2. Mice were individually placed on the central platform of the maze facing the open arm and were able to explore the maze for 5 min. In Trial 1, immediately after the exposure to the EPM, animals received microinjection of saline or histamine in the cerebellar vermis (0.54, 1.36, 2.72 and 4.07 nmol/0.1 μl). Twenty-four hours later (Trial 2), mice were re-exposed to the EPM under the same experimental conditions, but they did not receive any injection. Between subjects, the maze was thoroughly cleaned with 5% ethanol and a dry cloth.

2.6. Behavioral analysis

All sessions were video recorded by a digital camera linked to a computer in an adjacent room. Images were analyzed by a highly trained observer using X-PLO-RAT, an ethological analysis pack developed at the Laboratory of Exploratory Behavior USP/Ribeirão Preto [11]. Behavioral parameters were defined in a way that was consistent with previous studies [18,26] and included the following: the frequency of open- and enclosed-arm entries (OAE and EAE) (an entry was defined as the entry of all four of an animal's paws into an arm) and total time

Table 1

One-way ANOVA statistical results for the behavior of mice with no pharmacological treatment in Trial 1.

Behavioral measures	F	p
OAE	0.72	0.55
%OAE	0.34	0.80
OAT	0.99	0.41
%OAT	0.99	0.41
EAE	0.75	0.53
EAT	0.02	0.99
%EAT	0.02	0.99
CT	2.19	0.11
SAP	1.79	0.14
Head dipping	0.71	0.55
Immobility time	0.64	0.60

spent in the open arms (OAT), enclosed arms (EAT), and central area (CT). These data were used to calculate the percentage of open arm entries (%OAE = (open entries/open + enclosed entries) × 100); the percentage of time spent in the open arms (%OAT = (open time/300) × 100); and the percentage of time spent in the enclosed arms (%EAT = (enclosed time/300) × 100). The number of stretched-attend postures (SAP; exploratory posture in which the body stretches forward and then retracts to its original position without any forward locomotion), immobility time (stillness but some movement of the chest), and the frequency of head dipping (exploratory movement of head/shoulders over the sides of the maze) were also scored. Total SAP was considered a primary index of risk assessment and head dipping was considered an index of exploratory behavior [27].

2.7. Histology

At the end of testing, all animals received a 0.1 µl infusion of 1% methylene blue according to the microinjection procedure described above. The animals received an anesthetic overdose, their brains were removed and injection sites were verified histologically according to the atlas of Franklin and Paxinos [9]. Data from animals with injection sites outside the cerebellar vermis were excluded from the study. The final sample size of each cohort ranged between 11 and 16. Histology confirmed that a total of 70 mice had accurate cannula placements in the cerebellar dorsal vermis (Fig. 1) and that the sample sizes of the different dosage cohorts were as follows: saline ($n = 11$), 0.54 nmol histamine ($n = 15$), 1.36 nmol histamine ($n = 15$), 2.72 nmol histamine ($n = 16$) and 4.07 nmol histamine ($n = 13$).

2.8. Statistical analysis

All results were initially submitted to Levene's test for homogeneity of variance. The data were analyzed using a One-way Analysis of Variance (ANOVA) test. When differences were indicated by significant F values, they were identified by Duncan's multiple range tests. A p value of <0.05 was required for significance.

2.9. Ethics

The experiments carried out as part of this study were approved by the Animal Ethics Commission of the Federal University of Sao Carlos (CEEA 049/09), and they are in compliance with the norms of the Brazilian Neuroscience and Behavior Society (SBNeC), which are based on the US National Institutes of Health Guide for Care and use of Laboratory Animals.

3. Results

One-way ANOVA tests showed no significant differences between groups in Trial 1 for all the measures analyzed (Table 1). Therefore, the data were *pooled* because the animals had received no pharmacological treatment at that point. Fig. 2 illustrates the effects of microinjection of histamine on %OAE in the first and second sessions. The ANOVA test showed differences between trials ($F_{5,134} = 4.72$, $p = 0.0005$). The post hoc Duncan's test indicated that the mice that entered less often into the open arms in Trial 2 in comparison with Trial 1 were microinjected with saline ($p = 0.001$) and histamine at a dose of 0.54 nmol ($p = 0.02$). Moreover, the analysis showed a significant difference between the groups that had received saline and histamine at the dose of 4.07 nmol ($p = 0.007$).

Fig. 3 shows the %OAT for the first and second sessions. ANOVA analysis detected differences between sessions ($F_{5,134} = 6.17$, $p = 0.00036$) in %OAT. Post hoc analysis revealed that animals explored the open arms for a shorter time in the second trial

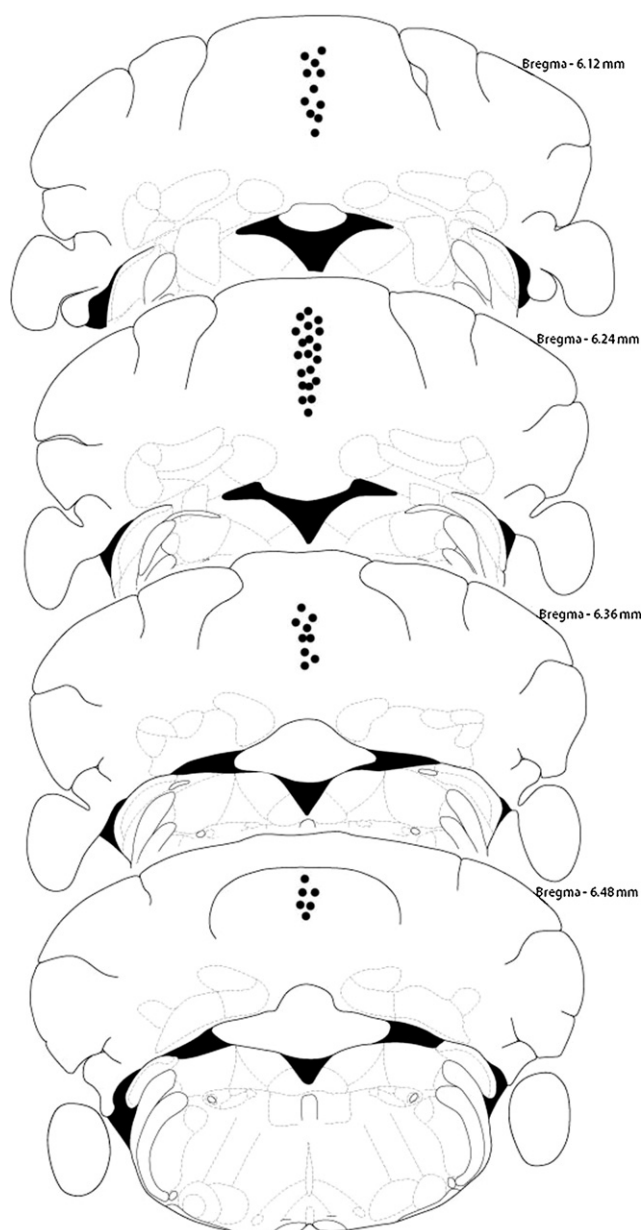


Fig. 1. Schematic representation (adapted from Franklin and Paxinos [9]) of sites of microinfusion into the cerebellums of mice. The number of points is smaller than the total number of mice because of overlaps.

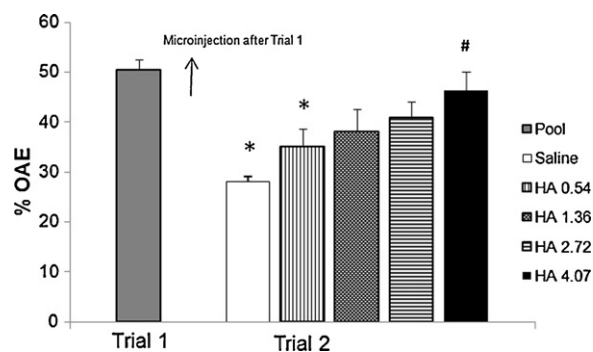


Fig. 2. Effects of microinjection of histamine (0.54, 1.36, 2.72 and 4.07 nmol) on the percentage of open arm entries (%OAE) in Trials 1 and 2 in the EPM. Data are presented as mean ± SEM. $n = 11$ –16. * $p < 0.05$ Trial 2 versus Trial 1; # $p < 0.05$ versus control (saline) in Trial 2.

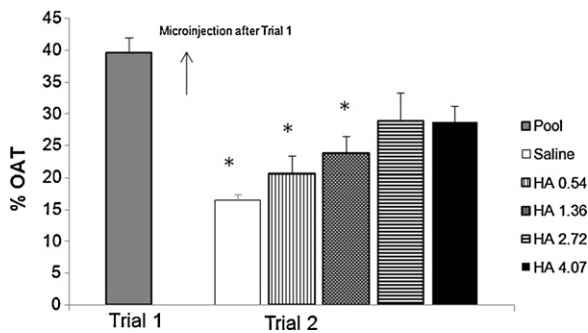


Fig. 3. Effects of microinjection of histamine (0.54, 1.36, 2.72 and 4.07 nmol) on the percentage of open arm time (%OAT) in Trials 1 and 2 in the EPM. Data are presented as mean \pm SEM. $n = 11$ –16. * $p < 0.05$ Trial 2 versus Trial 1.

when they had been microinjected with saline ($p < 0.001$), histamine at a dose of 0.54 nmol ($p = 0.005$), and histamine at a dose of 1.36 nmol ($p = 0.01$). For the groups that received histamine at doses of 2.72 nmol (%OAE: $p = 0.16$; %OAT: $p = 0.09$) and 4.07 nmol (%OAE: $p = 0.52$; %OAT: $p = 0.10$), there were no differences between trials in both measures.

Table 2 shows the results for all other behaviors. ANOVA analysis did not indicate differences in EAE ($F_{5,134} = 0.61$, $p = 0.11$). The treatment did not change the locomotor activity. ANOVA analysis revealed significant differences between trials in OAT ($F_{5,134} = 8.2$, $p = 0.000001$). Post hoc comparisons indicated that this difference was present for the groups microinjected with saline ($p < 0.001$), histamine at a dose of 0.54 nmol ($p = 0.002$), and histamine at a dose of 1.36 nmol ($p < 0.001$). There were also differences between sessions in the EAT ($F_{5,134} = 9.41$, $p < 0.00001$), %EAT ($F_{5,134} = 9.41$, $p < 0.00001$), CT ($F_{5,134} = 5.85$, $p = 0.000063$), and frequency of head dipping ($F_{5,134} = 10.39$, $p < 0.00001$). ANOVA analysis did not detect any significant differences between trials in OAE ($F_{5,134} = 1.86$, $p = 0.11$), immobility time ($F_{5,134} = 1.56$, $p = 0.18$), or total SAP ($F_{5,134} = 1.13$, $p = 0.34$).

4. Discussion

The main experimental finding of this study is that the microinjection of histamine into the cerebellar vermis impairs emotional memory consolidation in mice re-exposed to the EPM because open arm exploration did not decrease in the second session. In addition, the results indicate that there is a dose-dependent effect of histamine on the inhibition of memory.

The EPM is a test that assesses behavior associated with emotion because the behavior exhibited during the test arises from a con-

flict between the motivation to explore the maze and the natural tendency to avoid open spaces [4,18]. The EPM is one of the most widely used behavioral tests in research on anxiety [5,25]. Recently, it has become useful for understanding the biological basis of emotion related to learning and memory [3,16,41]. During the EPM test, the animal acquires information about safe and dangerous areas in the maze. Repeated testing in the EPM provides an index of memory acquisition and retention because there are experience-dependent changes in behavior. Studies have shown that exposure to a second trial causes animals to reduce their open arm exploration [7,8,12,16]. Recently, Galvis-Alonso et al. [10] observed a reduction in open arm exploration in the second trial when the interval between sessions was 9 or 33 days, which suggests that aversion to the open arms is preserved even 33 days after the first maze exposure.

Previous studies on emotional memory performed by our research group using Trial 1/2 protocol on the EPM revealed that histamine plays an inhibitory role on emotional memory [12,36]. Also, other studies concerning the histaminergic system and memory demonstrated learning and memory impairment when histamine was administered [2,15,47]. Nishiga et al. [20] showed that rats on a histidine-deficient diet exhibited reduced hippocampal histamine contents and improved eight-arm radial maze performance. The results of a study by Liu et al. [19], which used histidine decarboxylase knockout mice, indicated that a lack of histamine improves fear conditioning consolidation. Furthermore, according to Alvarez and Banzan [1], histamine treatment interferes with the consolidation of avoidance responses and impairs latency and memory efficiency.

The microinjection of histamine into the cerebellar vermis demonstrated that the cerebellar histaminergic system is involved in the process of consolidation of emotional memory. Several lines of evidence support the involvement of the cerebellar vermis in emotional learning [32]. Moreover, studies have evaluated the role of the cerebellum in emotional memory consolidation, provided important information about the role of the cerebellum in memory and explained how neural circuits are related to aversive memories. Sacchetti et al. [33] demonstrated that cerebellar vermis blockade caused amnesia when performed immediately after the recall of fear memories and that the combined inactivation of the cerebellum and amygdala elicited amnesia of strong fear behavior. Thus, according to the authors, the cerebellum influences long-term emotional memories, and due to the strong learning, the cerebellum acquires a durable fear representation, which is sufficient to support memory processes even in the absence of sites that are crucial for emotion, such as the amygdala.

It has been previously proposed that the amygdala and cerebellum are functionally interconnected during aversive learning

Table 2
Effects of microinjection of histamine (0.54, 1.36, 2.72 and 4.07 nmol) on the behavior of mice in Trials 1 and 2 in the EPM.

	Pool	Saline	HA 0.54	HA 1.36	HA 2.72	HA 4.07
OAE	8.2 \pm 0.4	6.6 \pm 1.4	6.3 \pm 0.9	6.0 \pm 1.2	7.7 \pm 0.8	9.6 \pm 1.2
OAT	119.0 \pm 6.9	49.3 \pm 9.9*	62.0 \pm 9.6*	53.6 \pm 12.9*	86.9 \pm 13.2	86.2 \pm 7.5
EAE	8.5 \pm 0.5	13.9 \pm 1.35	11.1 \pm 1.1	10.1 \pm 1.1	11.1 \pm 1.5	10.7 \pm 1.1
EAT	89.9 \pm 5.3	178.3 \pm 53.7*	166.2 \pm 14.1*	163.9 \pm 21.4*	134.3 \pm 14.5*	125.3 \pm 12.8*
%EAT	29.6 \pm 1.8	59.4 \pm 7.9*	53.4 \pm 5.7*	54.7 \pm 7.1*	44.8 \pm 4.8**	41.8 \pm 4.3**
CT	91.6 \pm 0.5	72.4 \pm 21.8	71.8 \pm 7.5*	82.5 \pm 17.5*	78.8 \pm 10.5*	88.4 \pm 9.6*
SAP	9.3 \pm 0.0	6.9 \pm 1.1	7.9 \pm 1.1	7.0 \pm 0.9	9.1 \pm 1.4	8.5 \pm 1.6
HD	5.9 \pm 0.9	0.5 \pm 0.1*	1.7 \pm 0.6*	2.0 \pm 1.1*	1.7 \pm 0.5*	1.8 \pm 0.9*
Immobility time	0.0 \pm 0.1	0.0 \pm 0.0	0.1 \pm 0.1	0.5 \pm 0.5	0.0 \pm 0.0	0.0 \pm 0.0

Pool (animals exposed to EPM with no pharmacological treatment); Saline (microinjected immediately after Trial 1); HA (histamine microinjected immediately after Trial 1 doses of 0.54 nmol; 1.36 nmol; 2.72 nmol and 4.07 nmol/0.1 μ l); OAE (number of open arm entries); OAT (time spent in the open arms); EAE (number of enclosed arm entries); EAT (time spent in the enclosed arms); %EAT (percentage of time in enclosed arms); CT (central platform time); SAP (frequency of stretched-attend postures); HD (frequency of head dipping); immobility time. Data are presented as mean values (\pm SEM). The second is the unit measure of time. * $p < 0.05$, Trial 2 versus Trial 1; ** $p < 0.05$, versus control (saline).

[17,40]. According to Sacchetti et al. [34], the vermis and amygdala may interact, and the vermal electrical stimulation modulates amygdala activity. These effects are mediated by both direct and indirect anatomical connections between the cerebellum and limbic areas as well as through the paleocerebellar projections to ascending catecholamine neurons of the locus coeruleus, the ventral tegmental area and the periaqueductal gray [34]. Therefore, the present results may be due to the effect of histamine on one of these cerebellar projections.

In conclusion, the results indicate that there is a dose-dependent inhibition of memory when histamine is injected into the cerebellar vermis. Therefore, it can be suggested that histamine in the cerebellum impairs the consolidation of emotional memory in mice.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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